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In-Vitro Toxicology Approach to Explore Nicotine Effects in Oral Mucosal Culture

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ABSTRACT

Objective: To measure the effect of in vitro nicotine on reconstituted oral mucosal culture and to study its effect after 5 minutes and 24 hours in normal healthy uninflammed oral mucosa.

Material and Methods: This observational study was conducted at Department of Oral Pathology, Barts and London Queen Mary University London to measure the effect of in vitro nicotine on reconstituted oral mucosal model. The reconstituted human epithelium model was used and was supplied by Skin Ethic Laboratories, Nice, France. Cells viability was assessed by MTT assay. Working solutions (10µM, 100µM, 1mM, and 10mM) of nicotine were primed from 2.5M stock solution (Sigma,UK). The effect of nicotine was studied after 5 minutes and 24 hours in normal healthy uninflamed oral mucosa.

Results: It was found that that application of nicotine after 5 minutes and 24 hours' treatment on uninflamed oral mucosal model and did not significantly affect the viability at all concentrations used.

Conclusion: Nicotine did not show any effect on uninflamed mucosa and also had no momentous effect after 5 minutes and 24 hours respectively. Further workup on proteomics and genomics is suggested to confirm our observations.

Key words: Nicotine, Oral mucosa, Tobacco, Viability of cells.

Author`s Contribution
[1]Active participation in active methodology, Interpretation and discussion [2]Synthesis and Planning of the research, Conception, Review the Study, [3,4] Review and paper writing

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Introduction

Tobacco use is regarded as a major cause morbidity and mortality and its extensive use has potentially significant and negative effects on oral and systemic health. The genus tobacco comes from a source named after Jean Nicot, a French ambassador that is being credited for the shipment of tobacco from Portugal to Paris in the year 1560.[1] The prevalence of smoking in countries like Western Europe, Australia, and the United States and the developing world is rising.[2] Mackay and Eriksen in 2002 reported that tobacco smoking has serious and adverse health consequences in all of the countries of the world irrespective of socio-economic status.[3] People consume variety of tobacco goods which can be either chewed, smoked or sniffed.[4] Products that are consumed could be

smoked such as cigarettes, cigars, pipe tobacco or can be consumed smokeless as snuff and chewing tobacco.[5]

Cigarette smoking and tobacco usage are also associated with the development of other cancers, including cancer of the oesophagus, and the lungs.[6] Fatal diseases such as respiratory heart disease, chronic obstructive lung disease, stroke, pneumonia, aortic aneurysm and ischaemic heart disease are also associated with smoking. Non-fatal diseases like a peripheral vascular disease, cataracts, and periodontal disease are also suggested to be associated with smoking.[7] Both active and passive (environmental) cigarette smoking are the predisposing factors for cardiovascular morbidity and mortality.[5] Nicotine addiction results in exposure to various carcinogens and other bioactive compounds present in tobacco.[8] The risk of Renal cell carcinoma is increased with active smoking as compared to passive smoking.[9] Smoking during pregnancy is also associated spontaneous abortion, ectopic pregnancy and low birth weight babies limb reduction defects and various other congenital defects in children.

Nicotine (C10 H14 N2) is a naturally occurring alkaloid, obtained from the tobacco plant called Nicotina Tabacum present in the tobacco leaves and makes up about 5% of a tobacco plant by weight,[10] and is highly addictive.[11] It is now known that cigarette smoking is a result of addiction to nicotine and the amount of nicotine taken up by the people who use tobacco varies in each individual.[12] Nicotine, an important component of tobacco, is primarily. It is the addictive substance in tobacco and the main reason for the continuation of the use of tobacco-related products. Oral snuff and pipe tobacco contain concentrations of nicotine similar to cigarette tobacco, whereas cigar and chewing tobacco have only about half of the nicotine concentration of the cigarette tobacco. An average tobacco rod contains 10 to 14 mg of nicotine, and on average, about 1 to 1.5 mg of nicotine is absorbed systemically during smoking. It is suggested that 0.038-0.217M nicotine concentration is present in smokeless tobacco.[13] Cigarette smoking delivers rapid doses of nicotine into the brain, following each inhalation, 15-20 minutes is its distribution half-life with a terminal half-life of two hours in the blood. Nicotine has a penetrating effect on brain neurochemistry causing activation of nicotinic acetylcholine receptors and releases dopamine in the nucleus accumbens.[12] There are nicotinic acetylcholine receptors present in different regions of brain, autonomic ganglia, and the neuromuscular junction where from this nicotine acts and these are of two types, muscle and neuronal.[13] Nicotine is a type of psychomotor stimulant, and helps smokers to calm down when they are under stress and enables them to work more effectively and with a higher concentration.[12]

Nicotine is linked with many lesions inside the oral cavity.[2] It is recommended that nicotine could be correlated to the pathogenesis of oral white lesions.[14] Carcinogens in tobacco smoke are responsible for the development of oral diseases and cancer. Nicotine adds to the risk factors for cancer when it is nitrosated and in turn, makes carcinogenic tobacco-specific nitrosamines.[8]

In vivo studies revealed that topical application of 0.216M of nicotine to oral mucosa for two hours resulted in alterations in epithelium such as nuclear shrinkage and acantholysis.[15] In a study conducted by Chen et-al it was evident that when 6% nicotine alone or in a combination of other tobacco-specific nitrosamines such as 0.01% NNN, 0.01% NNK was applied on hamster cheek pouch and gastric mucosal epithelium, it showed signs of hyperplasia, hyperkeratosis and also moderate dysplasia.[16] They concluded that these changes may be associated with the development of squamous cell papillomas in animals. Du et al revealed that nicotine is more rapidly and completely absorbed through the mucosal membrane of non-keratinized regions like floor of the mouth that is the most permeable region, than through other parts of the mouth.[17] Nicotine has also been shown to increase the permeability of oral mucosa to N-nitrosonornicotine. Nitrosonornicotine is known to be a tobacco-specific carcinogen and is also suggested that 0.2% nicotine significantly increases the permeability of oral mucosa to NNN and 2% nicotine causes a further increase to this permeability.[17]

Material and Methods

This study was conducted at Department of Oral Pathology, Barts and London Queen Mary University of London. The reconstituted human epithelium model used in the study was supplied by SkinEthic Laboratories, Nice, France. The study focused on the effects of nicotine on an uninflamed stratified epithelial layer, when applied for a

period of 5 minutes and over 24 hours respectively. Tissue viability was assessed using a modified MTT assay. The reconstituted human epithelium is a three-dimensional tissue culture model derived from a buccal carcinoma and obtained by culturing transformed oral keratinocytes (TR146). The cells were seeded and cultivated in a specific medium for 14 days. The consequential culture came out to be a stratified epithelium with 5-7 cell layers of epithelium. Model cultures were transferred into a new 24 well culture plates (Costar, UK) containing 500µl maintenance medium per well and incubated for 2 hours at 37˚C in 5% CO2 in a humidified atmosphere. The cultures were transferred to a new 24 well plate containing fresh media for all experiments.

Working solution of nicotine + MTT ASSAY: Working solutions (10µM, 100µM, 1mM, and 10mM) of nicotine were set up from a 2.5M stock solution (Sigma,UK) and before use it was diluted in phosphate buffered saline (PBS). Viability assays were designed to quantify the proportion of cells that failed to survive under experimental conditions. In this study modified MTT assay was selected. The viability of exposed cultures was measured by the quantification of mitochondrial dehydrogenase activity using a modified MTT assay. MTT assay involves the use of a colour reaction as a measure of cell activity. MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a pale-yellow substrate, which is reduced to a dark blue insoluble formazan product when incubated with living cells. The amount of formazan uptake is measured using densitometry. At the end of the treatment period, the cultures were transferred into a new 24 well plate containing 300µl MTT solution (0.5mg/ml in PBS). The plate was wrapped in aluminium foil and incubated for 60 minutes at 37˚C in 5% CO2 in a humidified atmosphere. After incubation, the cultures were transferred to a new 24-well plate containing 750µl isopropyl alcohol per well and 750µl of isopropyl alcohol applied to the epithelial surface. The plate was carefully sealed with Parafilm®, to prevent evaporation, and then incubated for a further 2 hours at 37˚C to extract the formazan. The insert was then removed and any surface solution retained in the well. The plate was agitated gently in order to equilibrate the colour density. Expended cultures were discarded. Three 200µl aliquots from each well were transferred to a 96-well plate (Costar UK) and the optical density (OD) was determined using a Titertek Multiskan Plus plate reader. Epithelial viability was expressed as the absorbance at 570nm. The results for MTT assay were quoted as (mean ± standard deviation).

Results

In this study, the tissue viability was assessed using a modified MTT assay. The results for MTT assay were quoted as (mean ± standard deviation) and it was observed that nicotine had no significant effect after 5 minutes on the viability of uninflamed stratified epithelial layer at 10µM (88.98 ± 20.14), 100µM (77.12 ± 16.15), 1mM (87.82 ± 12.28) and 10mM (97.70 ± 14.77) concentrations, when compared to PBS control (100 ± 6.45), (Table 1).

Table 1: MTT results after 5 minutes nicotine treatment on uninflamed tissue (n=4)

Treatment	Viability	
	Mean	Standard deviation
Nicotine (10µM)	88.98	20.14
Nicotine (100µM)	77.12	16.15
Nicotine (1mM)	87.82	12.28
Nicotine (10mM)	97.70	14.77
Phosphate buffered saline	100	6.45

Moreover, nicotine had no significant effect after 24 hours on the viability of uninflamed stratified epithelial layer at 10µM (111.72 ± 18.21), 100µM (110.10 ± 13.30), 1mM (96.90 ± 10.44) and 10mM (120.29 ± 13.99) concentrations, when compared to PBS control (100 ± 12.78 %), (Table 2).

Table 2: MTT results after 24-hour nicotine treatment on uninflamed tissue (n=4)

Treatment	Viability	
	Mean	Standard deviation
Nicotine (10µM)	111.72	18.21
Nicotine (100µM)	110.10	13.30
Nicotine (1mM)	96.90	10.44
Nicotine (10mM)	120.29	13.99
Phosphate buffered saline	100	12.78

Thus, results from MMT assay showed that application of nicotine after 5 minutes and 24 hours' treatment on uninflamed oral mucosa did not significantly affect its viability at different concentrations used.

Discussion

Smoking has been associated with diseases of the lung, pulmonary airways, and oral cavity. Cytologic, genomic, and transcriptomic changes in oral mucosa correlate with oral preneoplasia, cancer, and inflammation (e.g. periodontitis). Most of the studies also suggest that nicotine contributes to inflammatory processes in the oral cavity, and plays a critical role in the development of oral white pre-malignant lesions, and could be associated with the development of cancer in different regions of the oral mucosa. Although this study provides an insight into the possible role of nicotine in the pathogenesis of tobacco-related lesions in the oral cavity, there is a potential complexity which may limit the significance of the findings. In vivo, mucosa adjacent to nicotine is a stratified squamous epithelium and the results hence, presumes that nicotine has to saturate through epithelium to exert its effect. Various studies have been conducted to understand the direct effects of nicotine on a reconstituted stratified squamous epithelium in vitro.[18] Recently, however, an in vitro reconstituted oral mucosa has become available. This system, thus, offers an alternative approach to evaluate the effect of nicotine on oral mucosa that may more closely reflect in vivo situation.

The aim of this study was to explore the outcome of nicotine on an uninflamed reconstituted oral mucosa. The results from viability studies suggested that nicotine treatment of uninflamed reconstituted oral mucosa after 5 minutes and 24 hours had no significant effect on the viability of the cells, only a subtle change in membrane integrity and there were no morphological changes in the appearance of the epithelium. Chang YC et al in their study showed that 4mM nicotine dose caused significant morphological alterations of microtubules and vimentin filaments which then lead to atypical changes and vacuoles formation within the oral fibroblasts.[19] In a study conducted by Schlage WK. et al, on oral organotypic epithelium models, after exposure to cigarette smoke (CS); CS was found to be associated with increased secretion of inflammatory mediators, induction of cytochrome P450s activity and overall weak toxicity. Using microarray technology, they also identified CS impact on xenobiotic metabolism-related pathways and alteration in inflammatory processes. They supported the use of oral organotypical tissue models for an impact assessment.[20] Another study was conducted by Filippo Zanetti et al, in which human gingival epithelial organotypic cultures were repeatedly exposed (3 days) for 28 min to CS. They also found a significant association of CS with proinflammatory mediators.[21]

Chang YC et al had also linked higher doses of nicotine to be responsible for causing irreversible changes in the morphological appearance of the cells.[19] In the present study, it was not possible to quantify the amount of mitrochondrial disruption by nicotine at the concentration range used. Further workup is required to confirm our observations. As no change in morphology was seen in these experiments, it might be better to look for electron microscopic changes. Moreover, DNA array technology can be used for effective detection of other cytokines release and any upregulated protein.[22] Through the genomic study, the indirect identification of protein products could be achieved by simply comparing normal/untreated tissue with diseased/treated tissue.

Conclusion

Nicotine concentration ranging from 10µM to 10mM had no significant effect on viability and morphology of the uninflamed oral mucosa. Short exposure to nicotine causes it to reduce while long-standing exposure caused it to amplify.

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